

# Hydrogenosomes

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## Contents

1	Introduction .....	176
2	The Hydrogenosomes of the Trichomonadina .....	178
3	The Hydrogenosomes of <i>T. pyriformis</i> .....	181
4	The Hydrogenosomes of <i>P. lanterna</i> .....	182
5	The Hydrogenosomes/Mitochondrion-like Organelles (MLOs) of <i>Blastocystis</i> sp. ....	184
6	The Hydrogenosomes of Chytridiomycete Fungi .....	186
6.1	Mitochondria versus Hydrogenosomes .....	187
6.2	Chytrids Perform a “Mixed Acid Fermentation” .....	187
6.3	The Hydrogenosomal Metabolism .....	189
6.4	The Role of the Hydrogenosomes in the Energy Metabolism of <i>Piromyces</i> sp. E2 .....	190
6.5	The Evolution of Hydrogenosomes from Fungal Mitochondria .....	191
7	The Hydrogenosomes of Anaerobic Ciliates .....	192
7.1	<i>N. ovalis</i> .....	194
7.2	In Silico Reconstruction of the Basal Hydrogenosomal Metabolism of <i>N. ovalis</i> ..	195
7.3	The Hydrogenosomes of Other Ciliates .....	196
7.4	Can the Methanogenic Symbionts Tell Us More about the Origin and Function of Ciliate Hydrogenosomes? .....	197
7.5	Evolutionary Aspects .....	198
8	Conclusions .....	200
	References .....	200

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**Abstract** “Hydrogenosomes” are mitochondrion-derived, double membrane-bounded organelles that produce hydrogen and ATP. These properties discriminate them from the likewise mitochondrion-derived “mitosomes” that produce neither hydrogen nor ATP. The only character that is most likely shared by mitochondria, hydrogenosomes, and mitosomes is their involvement in the Fe–S metabolism.

Hydrogenosomes and mitosomes are found in a broad spectrum of rather unrelated species of unicellular, anaerobic eukaryotes, suggesting that hydrogenosomes and mitosomes evolved repeatedly and independently in the various taxonomic groups. With the exception of two hydrogenosomes, all these organelles lack a genome and an electron transport chain, which makes it sometimes difficult to trace their origins back to their mitochondrial origins. However, genomic evidence, EST studies, and the analysis of the organellar metabolism clearly reveal both a mitochondrial descent and individual differences in the properties of the various organelles. In this paper, we describe the diversity of hydrogenosomes based predominantly on information that became available recently. We also pay attention to the fact that certain hydrogenosomes are found in close association with endosymbiotic methanogens.

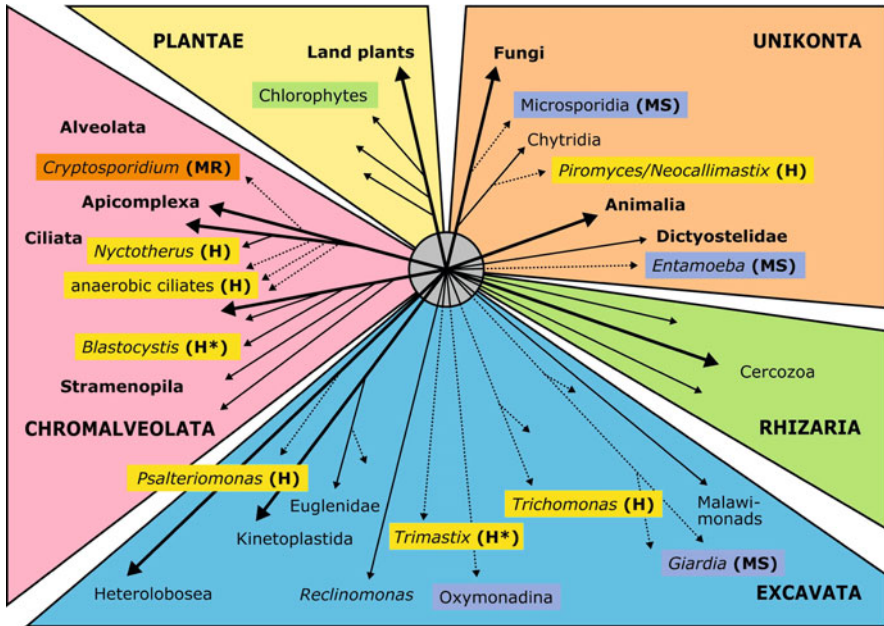
## 1 Introduction

It is now generally accepted that at least three different types of organelles evolved from one ancestral endosymbiont: mitochondria, hydrogenosomes, and mitosomes (Embley and Martin 2006; Hackstein et al. 2006; Howe 2008; van der Giezen 2009; Hjort et al. 2010). Mitochondria typically possess a genome that encodes components of an electron transport chain used in oxidative phosphorylation for the production of ATP. Usually, mitochondria are depicted as organelles that use oxygen as terminal electron acceptor in their process of ATP production. However, many mitochondria exist that can produce ATP without using any oxygen. The mitochondria of anaerobic eukaryotes produce ATP with the help of proton-pumping electron transport (i.e., oxidative phosphorylation), but they use terminal electron acceptors other than oxygen, such as fumarate (Tielens et al. 2002). In addition to anaerobic mitochondria, another type of anaerobic ATP-producing organelle exists, the hydrogenosome. Hydrogenosomes are double membrane-bounded organelles of a size of 0.3–2  $\mu\text{m}$  that are characterized by the production of hydrogen with the aid of a hydrogenase that donates electrons originating from the oxidation of substrates to protons (Müller 1993). In contrast to mitochondria and hydrogenosomes, mitosomes are not involved in the production of ATP. They are double membrane-bounded and present only in anaerobic eukaryotic organisms that lack mitochondria and hydrogenosomes. Mitosomes do not produce hydrogen. Their function is elusive and most of them share with mitochondria and hydrogenosomes only the presence of components of an iron–sulphur cluster synthesizing machinery (Tachezy and Dolezal 2007). Mitosomes and hydrogenosomes are found exclusively in anaerobic, unicellular organisms. Accumulating evidence suggests

that all these organelles evolved from mitochondrial precursors, accompanied by a loss of the organellar genome and the electron transport chain (Embley and Martin 2006; Hackstein et al. 2006; Tielens and van Hellemond 2007; van der Giezen 2009; Hjort et al. 2010). The discovery of a hydrogenosome with a mitochondrial genome and parts of an electron transport chain in the ciliate *Nyctotherus ovalis* discloses the existence of a hydrogen-producing mitochondrion, a “missing link” between hydrogenosomes and mitochondria (Boxma et al. 2005; Martin 2005). Notably, another missing link with a genome and an electron transport chain has been recently discovered in the unrelated Stramenopyle *Blastocystis* sp. (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008).

Hydrogenosomes and mitosomes are found in a broad spectrum of species, suggesting that hydrogenosomes and mitosomes evolved repeatedly in rather unrelated taxonomic groups (Fig. 1). Even within the Excavata, at least three different rather unrelated species with hydrogenosomes were identified, i.e., the heterolobosean amoeboflagellate *Psalteriomonas lanterna*, the preaxostylid flagellate *Trimastix pyriformis*, and the parabasalid flagellate *Trichomonas vaginalis* with its relatives *Tritrichomonas foetus*, *Monocercomonas* sp., and *Histomonas meleagridis*. In addition, *Giardia lamblia* (Excavata) is a species belonging to the diplomonads that hosts mitosomes. Among the Chromalveolata, various anaerobic ciliate species with hydrogenosomes evolved repeatedly from different aerobic ciliate progenitors (see below). One of them, *N. ovalis*, possesses a hydrogenosome with a genome (Akshmanova et al. 1998a; Boxma et al. 2005). Also, the Stramenopile *Blastocystis* sp. possesses a hydrogenosome-like, genome-bearing organelle (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008) and *Cryptosporidium* sp., which belongs to the apicomplexa and hosts a mitochondrion remnant (mitosome) that lacks a genome and a hydrogenase (Keithly 2008). Among the Unikonta, the anaerobic chytridiomycete fungi *Piromyces* sp. and *Neocallimastix* sp. possess hydrogenosomes (Müller 1993), while fungi-related Microsporidia such as *Encephalitozoon cuniculi* (Katinka et al. 2001), *Antonospora locustae* (Williams and Keeling 2005), and *Trachipleistophora hominis* (Williams et al. 2002) possess mitosomes. Mitosomes are also found in the rather unrelated species *Entamoeba histolytica* (Tovar et al. 1999) and *Mastigamoeba balamuthi* (Gill et al. 2007). These obviously diverse origins of the organelles (and hydrogenosomes in particular) strongly suggest that neither the various mitosomes nor the hydrogenosomes are the same. It is anticipated that the various organelles, even if they belong to the same type, are structurally and metabolically different. Lastly, on the basis of electron microscope studies, quite a number of potential hydrogenosome/mitosome-like organelles have been identified in anaerobes such as *Spironucleus elegans*, *Chilomastix cuspidata*, *Andalucia incarcerata*, *Lyromonas vulgaris*, *Monopylocystis visvesvarai*, *Sawyeria marylandensis*, *Carpediemonas membranifera*, *Dysnectes brevis*, *Vahlkamfia anaerobica*, *Percolomonas descissus*, *Postgaardi mariagerensis*, and *Breviata anathema* (Hampl and Simpson 2008).

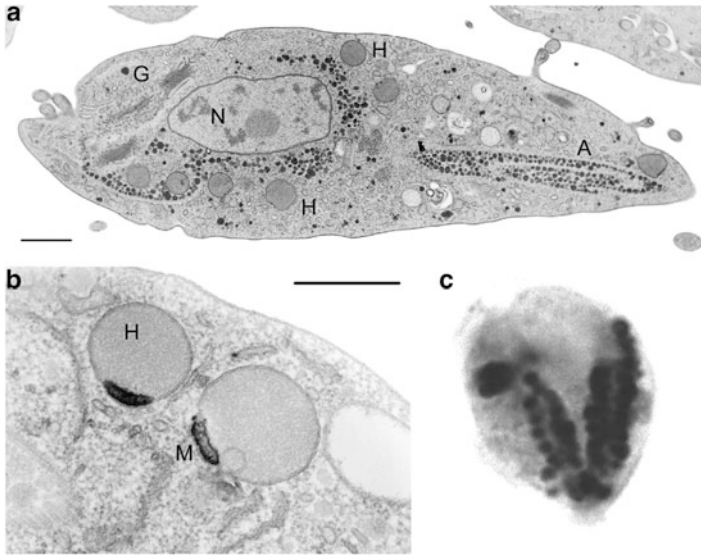
In this review, we focus on the hydrogenosomes and describe the different hydrogenosomes in the following in some detail.



**Fig. 1** Cartoon interpreting the evolution of mitochondria and related organelles (*H* hydrogenosomes, *MS* mitosomes, *MR* mitochondrial remnants). *H\** indicates that hydrogenase activity has not yet been demonstrated in *Blastocystis* and that the hydrogenase has not yet been localized to the organelle of *Trimastix*. The *solid lines* represent the evolutionary descent based on the presence of an organelle genome and the *broken lines* indicate the absence (loss) of a genome. The monophyly of the mitochondria has been derived from the phylogenetic analysis of more than 444 alpha-proteobacterial and 2061 mitochondrial genomes (February 2010). Since the branching order is not resolved so far, the cartoon indicates solely the common origin by the “origin” in the centre. A later loss of the organelle genome after the diversification of the hosts. The phylogenetic relationships between the hosts are still discussed controversially. Therefore, the smallest common denominator is used to display an unrooted “tree” of the most basic taxonomic arrangement agreed by most biologists: plantae; uniconata (animals and fungi); rhizaria; chromalveolata; excavata. No attempts were made to root this tree, nor to give any indication of a potential branching order. Certain green algae possess “normal” mitochondria, but express a plastidic hydrogenase under anaerobic conditions (chlorophytes). Substantial differences in metabolism have been established for the hydrogenosomes of *Trichomonas*, *Piromyces/Neocallimastix*, and the various ciliates. From Hackstein et al. (2006), modified

## 2 The Hydrogenosomes of the Trichomonadina

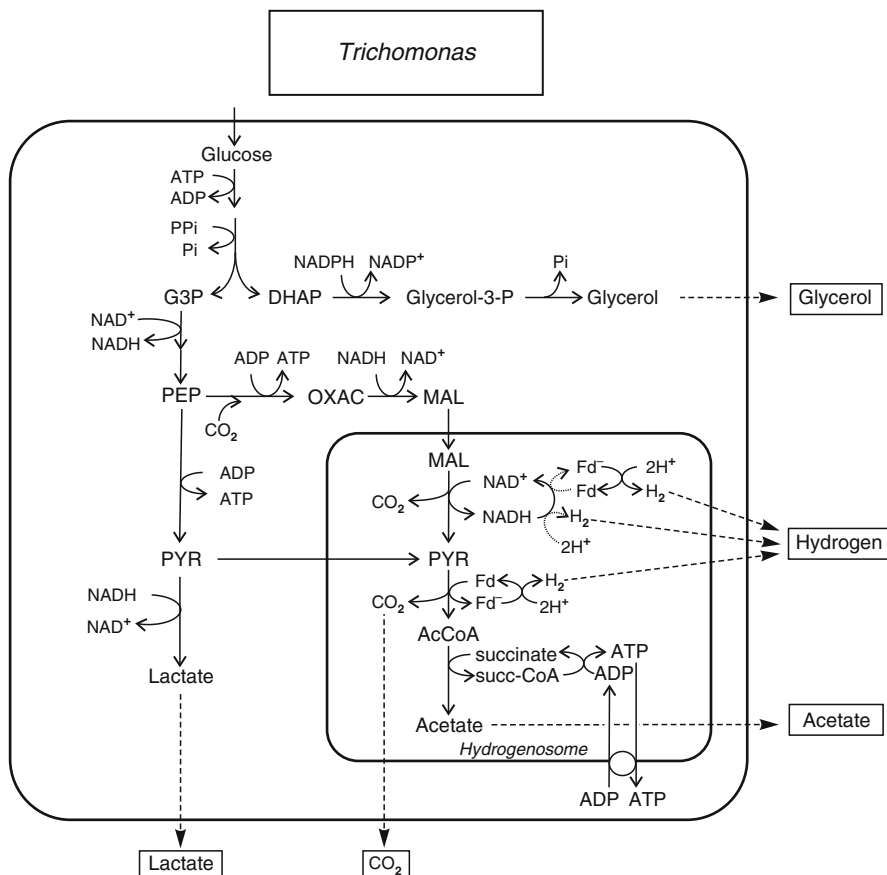
Trichomonads are microaerophilic, parasitic flagellates that belong to the Parabasalia. The hydrogenosomes of Trichomonads are the best-studied organelles of this kind. They were discovered in 1973 in *T. foetus*, 1974 in *Monocercomonas* sp., 1975 in *T. vaginalis*, and 2008 in *H. meleagridis* (Lindmark and Müller 1973, 1974; Lindmark et al. 1975; Mazet et al. 2008). These organelles are double



**Fig. 2** (a) Electron micrograph of *Trichomonas foetus*: seven hydrogenosomes (*H*) can be identified in the cytoplasm (*N* nucleus, *G* Golgi apparatus, *A* axostyl). (b) A higher magnification reveals that a double membrane surrounds the hydrogenosomes (*M* marginal plate). a and b were kindly provided by M. Benchimol, Rio de Janeiro. Bar: in a and b 1  $\mu\text{m}$ . (c) *Trichomonas vaginalis*, light microscopical picture of cell stained with BSTP following Zwart et al. (1988) to demonstrate hydrogenase activity; natural size approximately 10  $\times$  45  $\mu\text{m}$ . Courtesy C.K. Stumm, Nijmegen

membrane- bounded and about 0.3  $\mu\text{m}$  in diameter in *T. foetus* and *T. vaginalis* (Fig. 2), 0.3–0.6  $\mu\text{m}$  in diameter in *H. meleagridis* (Mazet et al. 2008), and up to 2  $\mu\text{m}$  in length in *Monocercomonas* (Benchimol 2008). They do not contain an organellar genome (Clemens and Johnson 2000). Most of the recent studies focussed on the human parasite *T. vaginalis*. In 2007, the complete (nuclear) genome has been published allowing a reconstruction of the hydrogenosomal metabolism that corroborates the earlier enzymatic studies (Carlton et al. 2007). In addition, the proteome of the hydrogenosomes has been analysed. Together with the genome data, the analysis of the proteome suggests that the hydrogenosome of *T. vaginalis* consists of at least 200 different proteins (Henze 2008). This is considerably less than the 700–800 proteins predicted for yeast mitochondria (Sickmann et al. 2003) and suggests a significantly lower complexity of the hydrogenosomes in comparison to mitochondria.

The metabolism of the hydrogenosomes of *T. vaginalis* has been described in detail by Carlton et al. (2007) and Hrdý et al. (2008); it will be summarized below. The trichomonad hydrogenosomes import pyruvate and malate (Hrdý et al. 2008). The latter is decarboxylated to pyruvate by a NAD-dependent malic enzyme inside the hydrogenosome. The initial step in the catabolism of pyruvate is the oxidative decarboxylation by the pyruvate:ferredoxin oxidoreductase (PFO) (Fig. 3) to



**Fig. 3** Energy metabolism of *Trichomonas vaginalis*. Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in boxes. Abbreviations: *AcCoA* acetyl-CoA; *DHAP* dihydroxyacetonephosphate; *Fd* ferredoxin; *G3P* glyceraldehyde-3-phosphate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate; *PYR* pyruvate; *Succ-CoA* succinyl-CoA [adapted from Carlton et al. (2007) and Hrdý et al. (2008)]

acetyl-CoA and CO<sub>2</sub>. The reduced ferredoxin is reoxidized by a [FeFe] hydrogenase; the genome of *T. vaginalis* encodes five hydrogenases with hydrogenosomal targeting signals. It is assumed that one or the other hydrogenase reacts directly with NADH, alternatively under the involvement of ferredoxin and the 24 kDa and 51 kDa proteins that are homologous to the corresponding subunits of a mitochondrial Complex I.

The next step in the catabolism of pyruvate is the formation of acetate from acetyl-CoA with the simultaneous transfer of the CoA moiety to succinate (Fig. 3). This reaction is catalysed by the enzyme ASCT (acetate:succinate CoA-transferase). The corresponding gene was not identified in the draft genome version.

However, recently, the gene and the enzyme have been identified and characterized in detail (van Grinsven et al. 2008).

The succinyl CoA synthetase (SCS, also known as succinate thiokinase) uses the energy-rich CoA bond for the generation of ATP/GTP from ADP/GDP. It is the only enzyme of *Trichomonas* also known from the TCA cycle of aerobic mitochondria. It regenerates succinate for the reaction with ASCT. It is unknown to date whether the acetate, which is formed by the action of ASCT, is excreted via diffusion or by a so far unidentified transporter.

ATP and ADP are exchanged by a member of the mitochondrial carrier family, HMP 31 (Tjaden et al. 2004). Besides the energy-generating pathway, several other metabolic pathways have been identified. Most importantly, several components of the Fe-S cluster synthesizing machinery were identified – a function that is shared with mitochondria, hydrogenosomes of *Blastocystis*, and several mitosomes (Burri et al. 2006; Tachezy and Dolezal 2007). In addition, a glycine-decarboxylase complex (GDC, also known as glycine cleavage system, GCS) has been found. It allows the formation of THF-CH<sub>2</sub> (Methylene tetrahydrofolate), which is a key compound in the C<sub>1</sub> metabolism. Also this enzyme complex is found in mitochondria and the hydrogenosomes of *Nyctotherus* and *Blastocystis*. Furthermore, several enzymes with a function in the protection against reactive oxygen species (ROS) have been identified. Lastly, quite a number of proteins (Hmp35, HPP, Sam50, Pam18, Mdj1, Mge, Hsp10, Hsp60, and Hsp70) involved in the import of proteins into the organelle were detected (Hrdý et al. 2008).

### 3 The Hydrogenosomes of *T. pyriformis*

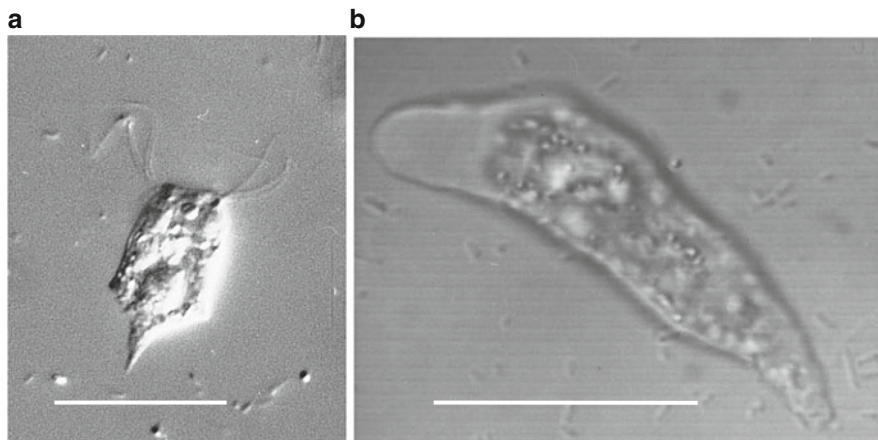
The anaerobic flagellate *T. pyriformis* belongs to the Preaxostyla. It is rather unrelated to the trichomonads although both genera belong to the supergroup Excavata. Electron microscopy has revealed the presence of double membrane-bounded organelles of a size of 0.3–1.2 µm, which, on the basis of their morphology, have been interpreted as hydrogenosomes (Brugerolle and Patterson 1997; O’Kelly et al. 1999; Simpson et al. 2000). Recently, a highly expressed [FeFe] hydrogenase has been identified in an EST study (Hampl et al. 2008). Also two enzymes involved in the maturation of [FeFe] hydrogenases were detected in that screen as well as a PFO. The localization of both enzymes has not yet been studied. Thus, it remains unclear as to whether the hydrogenase and the PFO are localized in the putative hydrogenosomes or in the cytoplasm. Notwithstanding, the presence of highly expressed hydrogenase and PFO genes suggests that the double membrane-bounded organelles are bona fide hydrogenosomes that have a metabolism similar to trichomonads. Notably, four proteins belonging to the GCS have been identified; a GCS is characteristic for mitochondria and also present in the hydrogenosomes of *Trichomonas*, *Blastocystis*, and *Nyctotherus*. Further support for a mitochondrial/hydrogenosomal nature of the organelles comes from the presence of three genes encoding mitochondrial carriers, three genes encoding components of an organellar

(mitochondrial) import machinery (TOM40, MPP, Hsp60), a lipoyl transferase, and a pyridine nucleotide transhydrogenase alpha. In addition, a gene encoding the TCA cycle enzyme aconitase was found that contrasts with the hydrogenosomes of *Trichomonas*, which do not possess TCA cycle enzymes except SCS (Carlton et al. 2007; Hampl et al. 2008; Hrdý et al. 2008).

Thus, there is no doubt that *T. pyriformis* hosts mitochondrion-derived organelles. The definitive classification of these organelles as hydrogenosome or mitochondrion depends on the localization of the hydrogenase and the PFO, i.e., if these enzymes are localized inside the organelles, they are hydrogenosomes, but if the enzymes are located in the cytoplasm, the organelles are mitochondria. Regardless of whether the organelles will be classified as hydrogenosomes or mitochondria, the available data clearly show that these organelles contain a unique set of proteins. Therefore, *T. pyriformis* harbours a unique version of anaerobic mitochondrion-related organelles.

#### 4 The Hydrogenosomes of *P. lanterna*

The third representative of the Excavata with hydrogenosomes is the microaerophilic amoeboflagellate *P. lanterna* (Broers et al. 1990; Broers 1992; Fig. 4). It belongs to the Heterolobosea, which are rather unrelated to both *T. pyriformis* and *T. vaginalis*. *P. lanterna* possesses full-fledged hydrogenosomes: hydrogen



**Fig. 4** Light microscopy of *Psalteriomonas lanterna*. (a) Flagellate stage of *Psalteriomonas lanterna* DIC-microscopy. At the apical side of the cell, two of the four flagella clusters can be seen. The globule in the centre of the cell is the hydrogenosomal complex. Courtesy C.K. Stumm, Nijmegen. (b) Amoeba stage of *Psalteriomonas lanterna*. CLS-microscope. Bars: 30 μm. Reproduced from de Graaf et al. (2009)

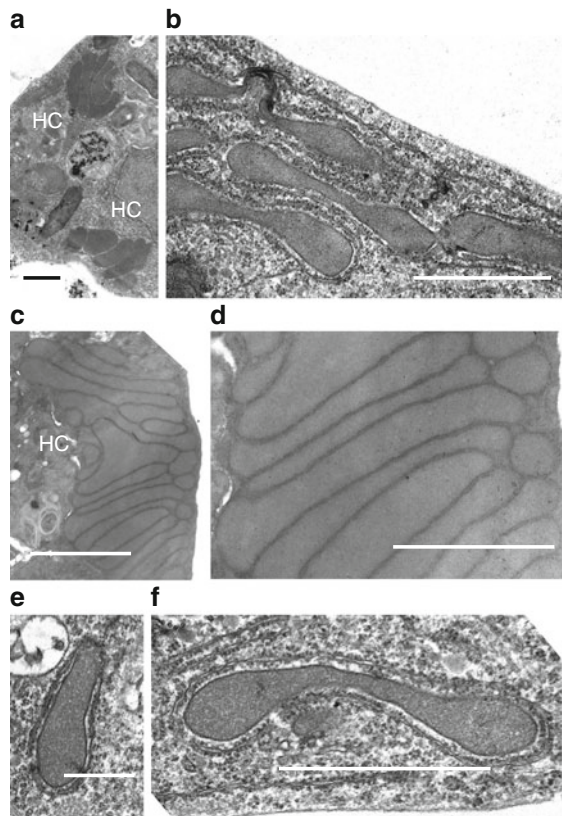


formation has been demonstrated, and hydrogenase activity was shown to be localized to the hydrogenosomes (Broers 1992).

Electron microscopy revealed the presence of two types of double membrane-bounded organelles (1) single, predominantly dumbbell-shaped organelles that are scattered in the cytoplasm and surrounded by 1–2 cisterns of rough ER and (2) stacks of up to 20, more or less sausage-shaped organelles, which are located in the centre of the cell (de Graaf et al. 2009; Fig. 5). At the isolation of *P. lanterna* from anaerobic sediments, these stacks of hydrogenosomes were “spiked” with methanogenic archaea (Broers et al. 1990), which were indicative of an intracellular source of hydrogen (and potentially other end products of the hydrogenosomal metabolism). The endosymbiotic methanogens were lost in the course of the prolonged in vitro culture of *P. lanterna*.

The cytoplasmic organelles are very similar to the mitochondria of certain related aerobic Heterolobosea because of their dumbbell shape and their close association with the rough ER (Fig. 5). Both types of hydrogenosomes are about 0.3–0.6  $\mu\text{m}$  in diameter and up to 3  $\mu\text{m}$  in length (de Graaf et al. 2009). There is no evidence for the presence of a genome.

**Fig. 5** Electron microscopy of the hydrogenosomes of *Psalteriomonas lanterna* flagellates. (a) Cell with two small stacks of hydrogenosomes. HC hydrogenosomal complex. (b) Group of dumbbell-shaped hydrogenosomes in the periphery of the cell. The hydrogenosomes are surrounded by cisterns of rough endoplasmic reticulum (rough ER). These organelles have been named “modified mitochondria” by Broers (1992). (c) Large stack of hydrogenosomes (HC). (d) Detail of the hydrogenosomal complex shown in c. (e) “Single” hydrogenosome surrounded by rough ER. (f) Dumbbell-shaped hydrogenosome (“modified mitochondrion”) Bars a–d, f: 1  $\mu\text{m}$ ; e: 0.5  $\mu\text{m}$ . Reproduced from de Graaf et al. (2009)

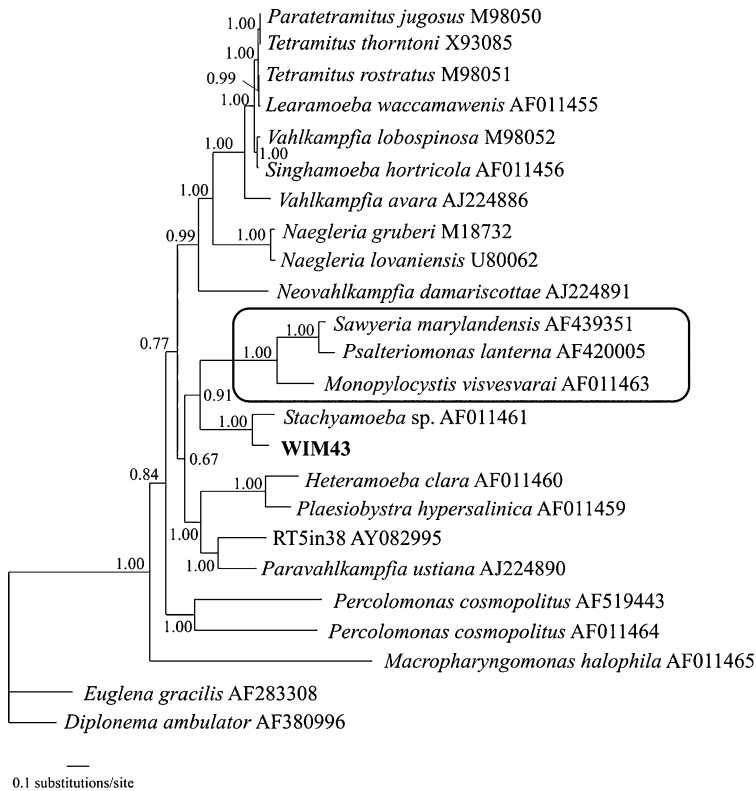


The analysis of about 480 ESTs allowed the identification of a number of hydrogenosomal/mitochondrial genes. Besides a [FeFe] hydrogenase of the long type that is phylogenetically related to the hydrogenases of anaerobic chytrids and certain green algae (de Graaf et al. 2009), a PFO with significant sequence similarity to the PFOs of *T. vaginalis* and *Blastocystis* sp. was found. In addition, a gene with sequence similarity to the 51 kDa subunit of a mitochondrial Complex I has been identified, which is phylogenetically related to the 51 kDa subunit of *T. vaginalis* (de Graaf et al. 2009). Furthermore, a member of the mitochondrial carrier family has been identified. Phylogenetic analysis revealed that this carrier does not cluster with the true mitochondrial AACs and not with the alternative ATP/ADP carriers of *Trichomonas*. Its phylogenetic position suggests that it is another type of an alternative AAC. Lastly, a Hsp60 and a PCCB (propionyl-CoA carboxylase beta) gene were found among the ESTs. Both genes are characteristic for mitochondria. In the phylogenetic analysis, both genes cluster with their homologs from the related aerobic heterolobosean amoeboflagellate *Naegleria gruberi*. Also the phylogenetic analysis of the 18S rRNA genes of *P. lanterna* and its relatives shows that the few anaerobic Heterolobosea cluster among their many aerobic relatives (Fig. 6; Weekers et al. 1997; O'Kelly et al. 2003; Moon-van der Staay et al. 2006).

The morphology of the hydrogenosomes and the genes that were identified in the EST screen prove the mitochondrial ancestry of the hydrogenosomes of *P. lanterna*. Although the number of genes studied is very small, it was possible to reconstruct a rudimentary scheme of the hydrogenosomal metabolism (de Graaf et al. 2009). The PFO decarboxylates pyruvate to acetyl-CoA and CO<sub>2</sub>. The electrons are transferred to the hydrogenase either with the aid of a so far unidentified ferredoxin or by the 51 kDa subunit. It is likely that ATP is formed in a similar way as in *T. vaginalis* and that the ATP is exported by the potential mitochondrial ATP/ADP carrier. Thus, the key enzymes of this metabolism are similar to those known from the hydrogenosomes of *Trichomonas* and *Trimastix*. However, the phylogenetic analysis has shown that the peculiar hydrogenosomes of *P. lanterna* derived from the mitochondria of its aerobic relatives among the Heterolobosea (Fig. 6) – clearly distinct from the descent of the hydrogenosomes of *Trichomonas* and *Trimastix* that do not have close aerobic relatives.

## 5 The Hydrogenosomes/Mitochondrion-like Organelles (MLOs) of *Blastocystis* sp.

The Stramenopile *Blastocystis* sp. is an anaerobic gut parasite that belongs to the supergroup Chromalveolata. It possesses double membrane-bounded organelles with both mitochondrial and hydrogenosomal properties. The organelles have been studied by enzymatic and molecular (EST) methods (Lantsman et al. 2008; Stechmann et al. 2008). These analyses had results that are sometimes controversial. For example, a [FeFe] hydrogenase has been identified among the ESTs and localized to the organelles with the aid of histocytochemistry using an antiserum



**Fig. 6** Bayesian tree of Heterolobosea based on nuclear SSU rRNA gene sequences using 1698 positions. Numbers at nodes represent the posterior probability. Anaerobic Heterolobosea are boxed. After Moon-van der Staay et al. (2006), modified

against the *Blastocystis* hydrogenase. Therefore, the organelles can be regarded as hydrogenosomes (Stechmann et al. 2008). However, enzymatic studies failed so far to provide evidence for hydrogenase activity (Lantsman et al. 2008). Also, the EST analysis provided evidence for the presence of a PFO. Enzymatic studies, however, did reveal pyruvate:NADP oxidoreductase (PNO) activity instead of PFO activity. PNO is as PFO, a strictly anaerobic enzyme that decarboxylates pyruvate. It has been found also in the mitochondrial remnants (mitosomes) of *Cryptosporidium* sp. and the mitochondria of *Euglena gracilis*. In *Blastocystis*, PNO decarboxylates pyruvate to acetyl-CoA and CO<sub>2</sub>; acetyl-CoA is metabolised to acetate by ASCT, an enzyme also present in hydrogenosomes. The CoA moiety is transferred to succinate that is recycled via a SCS (Lantsman et al. 2008; Stechmann et al. 2008). This is a pathway present in all hydrogenosomes studied up to now, and the genes ASCT and PFO in *Blastocystis* have significant sequence similarity to the homologous genes of *Trichomonas*.

The organelles host an incomplete TCA cycle. The EST studies provided evidence for SCS, succinate dehydrogenase (SDH), fumarase (Fum), and malate

dehydrogenase (MDH) genes, but the enzymatic studies revealed enzymatic activity of aconitase, isocitrate dehydrogenase, and  $\alpha$ -ketoglutarate dehydrogenase in addition to the SCS activity. A SDH activity was not observed, although the EST studies revealed the presence of all four subunits of a mitochondrial Complex II (Lantsman et al. 2008; Stechmann et al. 2008).

EST analysis and sequencing of the organellar genome (Perez-Brocail and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008) identified 16 genes encoding subunits of a mitochondrial Complex I. The sequencing of the organellar genome disclosed a typical mitochondrial genome with 41 genes – with the notable exception of genes encoding subunits of mitochondrial Complex III, IV, and V, which were lacking. Since an alternative oxidase (AO) has been found among the ESTs, it is likely that *Blastocystis* possesses an electron transport chain consisting of Complex I, Complex II, and an AO. This is in remarkable agreement with the electron transport chain in the hydrogenosomes of *N. ovalis*, which, however, lack typical AO activity (Boxma et al. 2005). It is likely that Complex I is functional and – in the absence of Complex III/IV – accounts for the transmembrane potential that has been observed in the organelles of *Blastocystis* (Stechmann et al. 2008).

The EST analysis provided also evidence for the presence of various components of an amino acid metabolism, including a GCS that is found in mitochondria and many hydrogenosomes. Also six components of a Fe–S assembly pathway have been found, and parts of an urea cycle.

Thus, the metabolism of the *Blastocystis* organelles has mitochondrial, but also many hydrogenosomal traits. The metabolism is a remarkable example of convergent evolution if compared with the hydrogenosomal metabolism of *N. ovalis*. It represents a blueprint for the adaptation to anaerobic environments.

## 6 The Hydrogenosomes of Chytridiomycete Fungi

Fungi form a very diverse group of eukaryotes belonging to the Unikonta. The majority of investigated fungi contain mitochondria and are capable of oxidative phosphorylation. On the other hand, there are anaerobically functioning chytridiomycete fungi that contain hydrogenosomes (Hackstein et al. 2008a).

Anaerobic chytridiomycete fungi are important symbionts in the gastrointestinal tract of herbivorous mammals. A flagellated rumen-dwelling organism, *Neocallimastix frontalis*, was described in 1975 by Colin Orpin (1975). Two years later, Orpin published a report showing that *N. frontalis* and two other anaerobes had cell walls that contained chitin, indicating that these rumen-dwelling organisms are fungi (Orpin 1977).

The diversity of the anaerobic chytridiomycete fungi is large and they are found in the gastrointestinal tract of nearly all large herbivores, ranging from ruminants such as cattle, sheep, goat, deer, and antelopes to the foregut-fermenting marsupials and camelids on the one hand, and hindgut-fermenting species such as horse, elephant, rhinoceros, mara (Patagonian hare), and capybara (“water pig,” the world’s

largest rodent) on the other. Anaerobic chytridiomycetes can be isolated from rumen fluid or faeces, and they are maintained in anaerobic culture, most of them as pure axenic cultures. In the rumen of cattle or sheep, these anaerobic fungi can be as frequent as  $7.6 \times 10^8$  thallus-forming units; in the faeces, there are still  $4.2 \times 10^4$  units per g dry weight (Trinci et al. 1994). Anaerobic chytrids are not truly host specific since it is possible to transfaunate various host animals with isolates from different hosts. On the other hand, the various isolates are not the same, even if collected from the same host species and assigned to the same chytrid species. The patterns of utilization of substrates and the metabolic properties are different from isolate to isolate (Trinci et al. 1994).

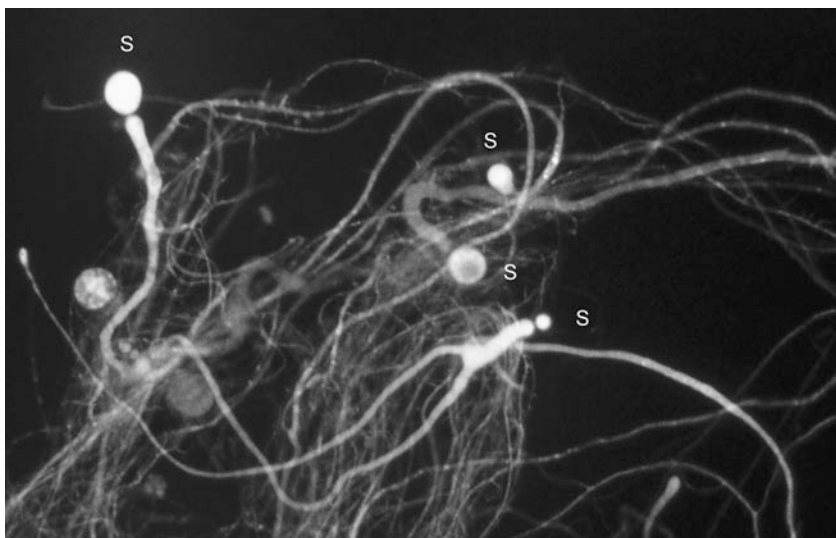
### 6.1 *Mitochondria versus Hydrogenosomes*

The majority of the cultured fungi belonging to the taxa Ascomycota, Basidiomycota, and Zygomycota contain mitochondria. These mitochondria host a genome of varying size, which characteristically encodes only a handful of proteins (Bullerwell and Lang 2005). This implies that the vast majority of the 700–800 mitochondrial proteins (Sickmann et al. 2003) is nuclear encoded, synthesized in the cytoplasm, and imported into the organelles. Interestingly, certain cultivars of mitochondriate species are able to maintain mitochondria in the absence of a mitochondrial genome. Such yeasts are known as “petites”; they are viable but respiration deficient and, consequently, incapable of growing on non-fermentable substrates (Contamine and Picard 2000). In this respect, these mitochondria are similar to the genome-less chytrid hydrogenosomes.

On the other hand, two natural isolates of fission yeasts, *Schizosaccharomyces japonicus* var *japonicus* and *S. japonicus* var *versatilis*, lack detectable cytochromes and are respiration deficient, but nevertheless retained fully functional mtDNA (Bullerwell and Lang 2005). These fission yeasts are considered to be an intermediate evolutionary stage in between respiratory-competent fungi and those that completely lack mitochondrial DNA. The mitochondria of these yeast species might be similar to the genome-containing hydrogenosomes of the anaerobic ciliate *N. ovalis* that are described in detail below. Therefore, these respiration-deficient mitochondria represent an evolutionary intermediate between classical mitochondria and the hydrogenosomes of the chytridiomycete fungi.

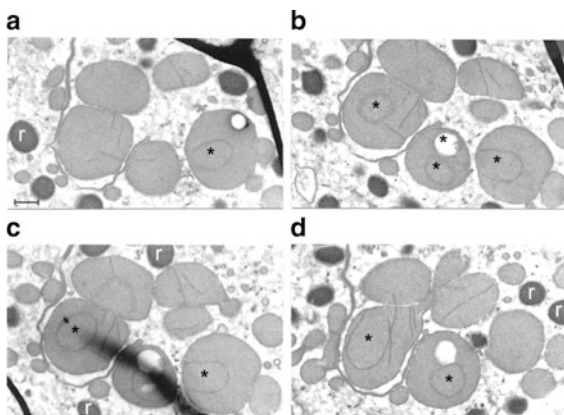
### 6.2 *Chytrids Perform a “Mixed Acid Fermentation”*

Notably, the members of the phylum chytridiomycota such as *Piromyces* (Fig. 7) and *Neocallimastix*, which possess hydrogenosomes, lack both mitochondria and an organellar genome (van der Giezen et al. 1997). These hydrogenosomes of chytrid fungi are double membrane-bounded compartments up to 1  $\mu\text{m}$  in size (Fig. 8) that



**Fig. 7** Epifluorescence micrograph of *Piromyces* sp. E2 originally isolated from the faeces of an Indian elephant. Magnification about  $\times 400$ . The organism was vitally stained with Rhodamine 123. S: young sporangia. Reproduced with permission from Hackstein et al. (2008a)

**Fig. 8** Electron micrographs of the hydrogenosomes of *Neocallimastix* sp. L2. Serial sectioning, **a–d**. Bar = 0.5  $\mu\text{m}$ ; *r* ribosome globules (Munn et al. 1988). Asterisk, internal vesicular structures of the hydrogenosomes. Reproduced with permission from Hackstein et al. (2008a)

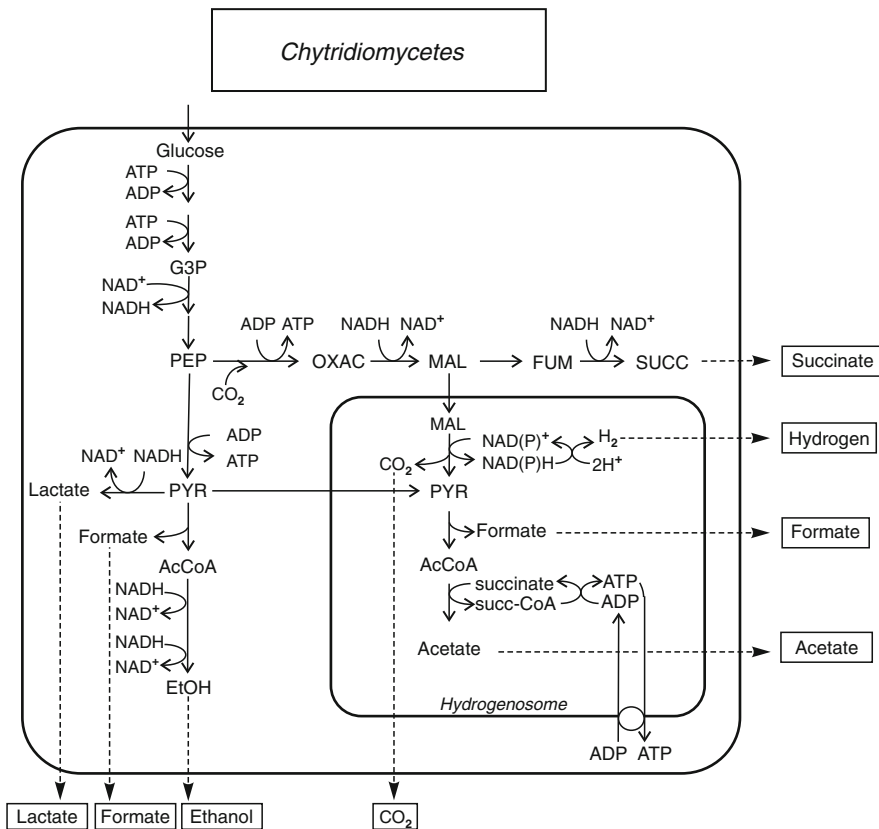


produce ATP by substrate-level phosphorylation together with hydrogen,  $\text{CO}_2$ , formate, and acetate as end products of the organellar metabolism (Marvin-Sikkema et al. 1993, 1994; Akhmanova et al. 1999; Hackstein et al. 2001; Voncken 2001). The intact organism produces succinate, lactate, and ethanol in addition when growing on cellulose, glucose, or fructose as a carbon source (Julliard et al. 1998). Such a “mixed acid fermentation” is very similar to bacterial mixed acid fermentations that are, for example, well known for facultative anaerobic enteric bacteria, such as *Escherichia coli*.

### 6.3 The Hydrogenosomal Metabolism

The hydrogenosomal metabolism has been studied in more detail in the chytridiomycetes *Piromyces* and *Neocallimastix*. Notably, the hydrogenosomes of these organisms are clearly different from those known of Trichomonads and anaerobic ciliates, structurally (Fig. 8) and metabolically. Most importantly, the hydrogenosomes of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 contain PFL as key enzyme (Akhmanova et al. 1999), and not PDH (as in *N. ovalis*) or PFO (as in *T. vaginalis* and many other anaerobic organisms). Accordingly, PFO is lacking as the analysis of a large collection of ESTs reveals.

As discussed above, these chytridiomycetes produce formate, acetate, succinate, lactate, ethanol, hydrogen, and carbon dioxide (Fig. 9). However, the ratio of these



**Fig. 9** Energy metabolism of chytridiomycetes. Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in boxes. Abbreviations: *AcCoA* acetyl-CoA; *EtOH* ethanol; *FUM* fumarate; *G3P* glyceraldehyde-3-phosphate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate; *PYR* pyruvate; *SUCC* succinate [adapted from Boxma et al. (2004)]

excreted end products is not constant, as it was shown that the growth of *Piromyces* sp. E2 in the presence of increasing concentrations of fructose is accompanied by changes in the fermentation pattern (Boxma et al. 2004). Increasing the fructose concentration from 0.1 to 0.5% resulted in a threefold increase in degradation of this substrate to end products. It is remarkable that the relative fluxes of fructose degradation through the various pathways were not constant during changing fructose concentrations. Although the absolute amounts of hydrogen formed in the incubations during growth at these increasing concentrations of fructose remained constant, the relative flux of malate into the hydrogenosomes and hence the relative flux to hydrogen decreased from 47 to 15% (Boxma et al. 2004). In contrast, the relative fluxes in the formation of the cytosolic end products lactate, ethanol, and succinate increased several fold. These observations show that increasing amounts of a fermentable carbon source result in an increased metabolism without an increased production of hydrogen. This implicates a relative shift from a hydrogenosomal carbon metabolism to a cytosolic one.

Metabolic experiments using labeled glucose indicated that an incomplete TCA cycle operates in the reductive mode allowing the formation of succinate from oxaloacetate via a malate intermediate (Fig. 9). Since the formation of significant amounts of labeled CO<sub>2</sub> could be excluded while formate and acetate plus ethanol were formed in a 1:1 ratio, it must be concluded that PFL and not PFO or pyruvate dehydrogenase (PDH) play the central role in the hydrogenosomal metabolism (Boxma et al. 2004). Moreover, experiments with isolated hydrogenosomes of *Piromyces* have shown that acetate and formate are formed in equimolar amounts confirming the activity of PFL in the hydrogenosomes (Akhmanova et al. 1999).

#### **6.4 The Role of the Hydrogenosomes in the Energy Metabolism of *Piromyces* sp. E2**

The observation that the hydrogenosomal PFL and the cytoplasmic ADHE are the key enzymes in the degradation of carbohydrates by anaerobic chytrids reveals that the metabolism of these hydrogenosomes is fundamentally different from the hydrogenosomal metabolism in both trichomonads and *N. ovalis*-like ciliates. Obviously, anaerobic chytrids chose their own way to adapt to anaerobic environments by evolutionary tinkering. The metabolic scheme displayed in Fig. 9 shows a generalized metabolism involving substrate-level ATP formation with the aid of ASCT and SCS. A quantitative analysis revealed that (1) PFL must be present and (2) that under certain conditions, hydrogen formation can become marginal (Boxma et al. 2004). The evolutionary strategy of chytrids apparently tends to avoid the formation of reduction equivalents by using PFL instead of PFO or PDH (Akhmanova et al. 1999; Hackstein et al. 1999, 2006; Voncken 2001).

The major role of the chytrid hydrogenosomes seems to be the generation of ATP by substrate-level phosphorylation. The presence of PFL in the absence of



hydrogenosomal ADHE most probably directs all organellar pyruvate into substrate-level ATP formation. A possible presence of ADHE inside the hydrogenosomes would compromise this function of the hydrogenosome as an energy-generating organelle. In the cytoplasm, however, ADHE might allow regulation of PFL activity, thus saving pyruvate (and its metabolites) for anabolic pathways. A partial TCA cycle with links to anabolic pathways operates in the cytoplasm (Akhmanova et al. 1998b). This hypothesis is supported by the observation that several mitochondrial enzymes, which are involved in anabolic reactions, e.g., malate dehydrogenase, aconitase, isocitrate dehydrogenase, and acetohydroacid reductoisomerase, have been retargeted to the cytoplasm in *Piromyces* sp. E2 (Akhmanova et al. 1998b; Hackstein et al. 1999). Consequently, compartmentalization of the energy metabolism seems to enhance the possibilities for regulation of the metabolic pathways of this organism.

## 6.5 *The Evolution of Hydrogenosomes from Fungal Mitochondria*

Using 18S rDNA phylogenies or the phylogenies of mitochondrial genes from aerobic chytrids, a monophyletic origin of all chytrids becomes evident (Bullerwell and Lang 2005). There is no doubt about a fungal origin of the chytrids – regardless as to whether they are thriving in oxic or anoxic environments. The aerobic representatives possess mitochondria: phylogenetic analysis of their nuclear and mitochondrial genomes reinforces their fungal origin (Bowman et al. 1992; Paquin et al. 1995; Paquin and Lang 1996). Also an analysis of biochemical and morphological traits consistently establishes a close relationship between chytrids and other fungi (Ragan and Chapman 1978). Akhmanova et al. (1998b) demonstrated that several enzymes of mitochondrial origin, which lack putative targeting signals, were retargeted to the cytoplasm (in active form) in the hydrogenosome-bearing chytrid *Piromyces*.

Chytrid hydrogenosomes look rather different from the pictures of mitochondria in textbooks (Fig. 8). However, electron microscopical analysis revealed a structure resembling the ultrastructure of mitochondria from particular diseased human patients (Frey and Mannella 2000; Hackstein et al. 2001; Voncken et al. 2002). Also, the relict mitochondrion (mitosome) of *Cryptosporidium parvum* looks very similar (Keithly et al. 2005). Apparently, in these cases, the inner membrane undergoes a derangement in the mechanism that normally stabilizes the crista junctions (Mannella 2006).

Because of their intrinsic function in the organelle, ADP/ATP carriers (AACs) and chaperonins are the best indicators for the phylogenetic analysis of an organelle of mitochondrial origin. Phylogenetic analysis of the AACs and chaperonins of anaerobic chytrids unequivocally revealed a fungal mitochondrial ancestry (Voncken 2001; Voncken et al. 2002; van der Giezen et al. 2002, 2003). Moreover, the spectrum of responses against the various inhibitors is quite specific and

differentiates these AACs clearly from other adenine transporters – regardless as to whether these transporters are from mitochondrial or hydrogenosomal origin (Hackstein et al. 2006). While the AACs are eukaryotic “inventions” that allowed the exploitation of the ATP formed inside the organelle after the organelle formation, the chaperonins tend to trace the ancestry of the organelle back to the endosymbiont that gave rise to the mitochondrion. Also, the phylogenetic analysis of Hsp 60 and the Hsp 70 clearly reveals a clustering with their fungal mitochondrial relatives and not with the alpha-proteobacterial cluster (Hackstein et al. 1999; Voncken et al. 2002; van der Giezen et al. 2003).

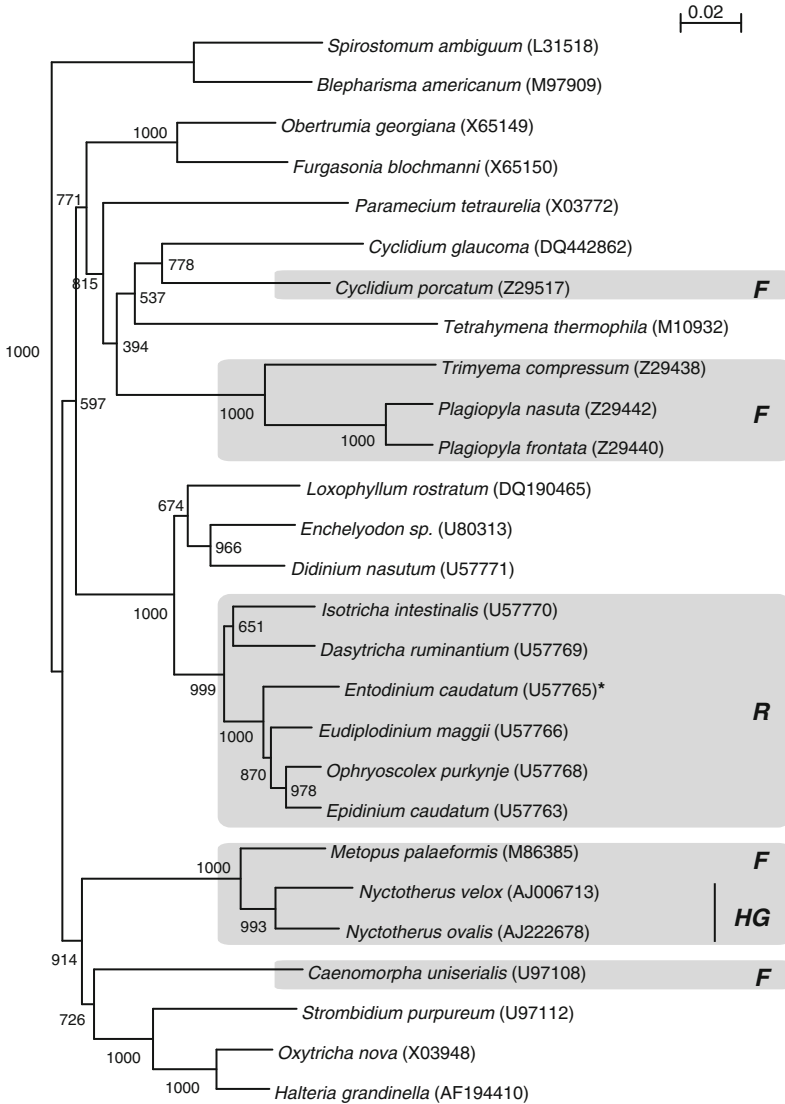
Genomic analyses of the hydrogenosomal enzyme succinyl-CoA synthetase, SCS, (Dacks et al. 2006) and two additional hydrogenosomal enzymes involved in arginine biosynthesis (Gelius-Dietrich et al. 2007) further confirm the fungal mitochondrial origin of the *Neocallimastix* hydrogenosome. Most of the other hydrogenosomal genes have not been identified so far.

We now know that the anaerobic chytrids comprise many species that are integral in the rumen ecosystem and crucial in the digestion of plant material to simple sugars. Moreover, they produce hydrogen needed for the growth of methanogenic bacteria [reviewed in Williams et al. (1994)]. However, there is no evidence for endo- or episymbiotic associations between anaerobic chytrids and methanogenic archaea. Notwithstanding, co-culture of chytrids and methanogens has profound effects on the overall metabolism of the chytrids (Marvin-Sikkema et al. 1990).

## 7 The Hydrogenosomes of Anaerobic Ciliates

Ciliates represent an extremely species-rich, monophyletic group of highly complex unicellular eukaryotes. They are characterized by a nuclear dimorphism and rather complex patterns of morphologically distinct cortical cilia. Most of the ciliates thrive in aerobic environments and possess mitochondria, but anaerobic species evolved in at least 8 of the 22 orders of ciliates as classified by Corliss (Corliss 1979; Fenchel and Finlay 1995). Certain ciliates in seven of these eight orders possess “hydrogenosomes” (Hackstein et al. 2008b; Fig. 10). However, the identification of many of these hydrogenosomes was based solely on the presence of intracellular methanogenic archaea. Such a symbiotic association is indicative of an inter-species hydrogen transfer and could reveal the presence of intracellular hydrogen sources, i.e., hydrogenosomes (Hackstein et al. 2002).

The development of fluorescence microscopy, electron microscopy, cytochemistry, and techniques for cellular fractionation allowed the discovery of hydrogenosomes in free-living anaerobic ciliates such as *Plagiopyla*, *Trimyema*, and *Metopus* (van Bruggen et al. 1983, 1984, 1986; Goosen et al. 1988, 1990; Zwart et al. 1988; Finlay and Fenchel 1989; Fenchel and Finlay 1995, 2010; Biagini et al. 1997; Shinzato and Kamagata 2010). Hydrogenosomes were also identified in ciliates thriving in the gastrointestinal tract of ruminants and marsupials (e.g., *Isotricha*, *Dasytricha*, *Epidinium*, *Eudiplodinium*, *Polyplastron*, and *Amylovorax*)



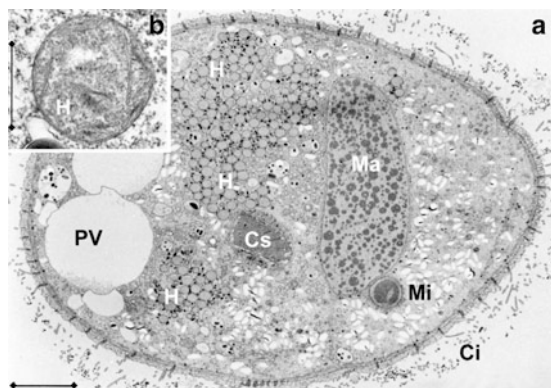
**Fig. 10** Neighbour-joining phylogenetic tree of 18S ribosomal RNA of ciliates. Ribosomal RNA sequences were aligned using Clustal X (Jeanmougin et al. 1998) and phylogenetic trees were prepared by neighbour-joining (Saitou and Nei 1987). Shown are the accession numbers of used sequences and the bootstrap values for 1,000 independent analyses. Shaded boxes indicate anaerobic ciliates with hydrogenosomes, whereas all other ciliates contain mitochondria and function aerobically. The natural habitat of the hydrogenosome-containing ciliates is indicated by the following abbreviations: *F* free living; *HG* hindgut; *R* rumen. Ciliate species that might possess mitosomes instead of hydrogenosomes are indicated by an asterisk. Reproduced with permission from Hackstein et al. (2008b)

(Vogels et al. 1980; Snyers et al. 1982; Yarlett et al. 1981, 1982, 1983, 1984, 1985; Lloyd et al. 1989; Paul et al. 1990; Ellis et al. 1991a,b,c; Cameron and O'Donoghue 2002a). They were also found in *N. ovalis* that lives in the hindgut of cockroaches (Gijzen et al. 1991; Akhmanova et al. 1998a; Boxma et al. 2005). Figure 10 shows the distribution of hydrogenosomes and mitochondria in the various orders of ciliates. All these hydrogenosomes are surrounded by a double membrane, and under optimal fixation conditions, in a number of ciliate species, cristae-like protrusions can be seen in these organelles, and in that way they clearly resemble mitochondria (Fig. 11).

## 7.1 *N. ovalis*

*Nyctotherus* species (Armophorea) are anaerobic, heterotrichous ciliates with hydrogenosomes that thrive in the intestinal tract of cockroaches, millipedes, frogs, and reptiles. *N. ovalis* from the hindgut of cockroaches is the only species that has been studied in more detail (van Hoek et al. 1998, 1999, 2000b). Notably, the presence of a mitochondrial genome has been demonstrated in the hydrogenosomes of *N. ovalis* (Akhmanova et al. 1998a; van Hoek et al. 2000a; Boxma et al. 2005). This genome was shown to be a typical mitochondrial genome of ciliate origin (Boxma et al. 2005). This ciliate origin is reinforced by the analysis of some 90 genes that encode mitochondrial proteins (Boxma et al. 2005; Ricard 2008, de Graaf et al. unpublished, see below).

Metabolic studies revealed that a small part of the glucose was degraded to typical end products of a glycolytic fermentation: approximately 24% of the degraded glucose was excreted as lactate and 5% as ethanol (Boxma et al. 2005). The major part of the glucose was degraded via the hydrogenosomes to acetate and succinate. Those studies showed that *N. ovalis* does not use a complete TCA cycle for the degradation of glucose and does not use pyruvate formate lyase (PFL) activity in its pyruvate metabolism, as is the case in hydrogenosomes of anaerobic



**Fig. 11** Electron micrograph of *N. ovalis* (a) with a close up view of a hydrogenosome (b). Bar in a: 10  $\mu\text{m}$ , bar in b: 0.5  $\mu\text{m}$ . H hydrogenosomes; Ma macronucleus; Mi micronucleus; Cs cystostome; PV pulsating vacuole. Reproduced with permission from Hackstein et al. (2008b)

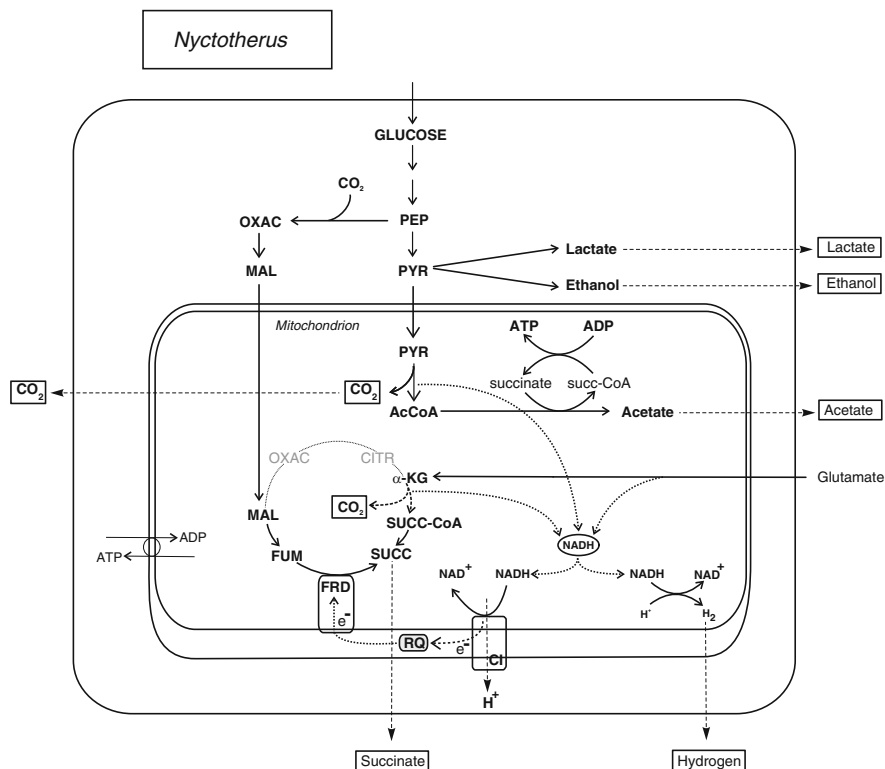
chytrids. The product of glycolysis in the cytosol, pyruvate, is apparently either converted into lactate or ethanol or transported into the hydrogenosome to be converted into acetate or succinate. For the production of acetate, this pyruvate is decarboxylated by a pyruvate dehydrogenase complex (PDH) and not by a PFO (Boxma et al. 2005). The excretion of significant amounts of succinate indicated that endogenously produced fumarate is used as a terminal electron acceptor. Protons act as another hydrogenosomal electron acceptor, which results in the formation of hydrogen. Fumarate reduction is most likely catalysed by a membrane-bound fumarate reductase (an anaerobically functioning variant of Complex II), coupled to Complex I of the electron transport chain via quinones. Consistent with the biochemical/biophysical requirements (Tielens et al. 2002), small amounts of rholoquinone 9 and menaquinone 8 were detected, whereas ubiquinone 7 and 8 (which are found in large amounts in the aerobic ciliates *Euplotes* and *Tetrahymena*, respectively) were not detected in *N. ovalis* (Boxma et al. 2005).

## 7.2 *In Silico Reconstruction of the Basal Hydrogenosomal Metabolism of N. ovalis*

The significance of these experimental data might be circumstantial without molecular support (Boxma et al. 2005). Genes for all four subunits of a PDH are present and are expressed. In addition, a gene was detected for acetyl-CoA synthase, an enzyme for the production of acetate from acetyl-CoA, and also several genes, which are predicted to encode enzymes of the TCA cycle, i.e., malate dehydrogenase, succinate dehydrogenase (2 subunits), succinyl-CoA synthetase, and alpha-ketoglutarate dehydrogenase (1 subunit). Thus, basically the core energy metabolism of a typical ciliate mitochondrion was detected, albeit in an anaerobic version (Fig. 12). In fact, *N. ovalis* contains hydrogen-producing mitochondria (Boxma et al. 2005).

There is no evidence for genes encoding components of mitochondrial Complexes III and IV. Notably, these complexes are also absent in the electron transport chains of anaerobic mitochondria and the hydrogenosomes of *Blastocystis* (Tielens et al. 2002; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Therefore, it is likely that these hydrogenosomes gain their energy by the generation of a PMF through proton pumping by the mitochondrial Complex I. Of the subunits of a mitochondrial Complex I, 12 out of the 14 subunits that form the core of a bacterial Complex I were cloned and sequenced until now (de Graaf unpublished). Accordingly, imaging studies using inhibitors and fluorescent dyes not only demonstrated the presence of a functional Complex I in these hydrogenosomes but also indicated the absence of functional Complexes III and IV and the absence of a plant-like alternative terminal oxidase (Boxma et al. 2005).

Also, no homologs of an F<sub>0</sub>F<sub>1</sub>-ATP synthase have been discovered so far, as in the organelles of *Blastocystis* and *Trichomonas* (Carlton et al. 2007; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008).



**Fig. 12** Speculative metabolic schemes of the main pathways in carbohydrate metabolism in the ciliate *Nyctotherus ovalis*. End products are in boxes. Abbreviations: *AcCoA* acetyl-CoA; *CI* Complex I; *FRD* fumarate reductase; *FUM* fumarate;  $\alpha$ -*KG*  $\alpha$ -ketoglutarate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate carboxykinase; *PYR* pyruvate; *RQ* rhoquinone; *SUCC* succinate; *SUCC-CoA* succinyl-CoA

In addition, components of a mitochondrial amino acid metabolism were identified, including a GCS. Moreover, components of fatty acid metabolism, an AAC, several members of the mitochondrial solute carriers family, a malate-oxoglutarate translocator, components of a mitochondrial protein import and processing machinery, components of a protein synthesizing machinery, and proteins belonging to a ROS defence systems were found. Several proteins originated from lateral gene transfer (Ricard 2008). Thus, the hydrogenosome of *N. ovalis* is not simply a rudimentary mitochondrion. It is a highly specialized organelle of considerable complexity.

### 7.3 The Hydrogenosomes of Other Ciliates

Metabolic studies have also been carried out on the hydrogenosomes of rumen ciliates such as *Dasytricha*, *Isotricha*, *Epidinium*, and *Eudiplodinium*. All rumen ciliates form a monophyletic group (Fig. 10; Strüder-Kypke et al. 2006), but not all

of them possess hydrogenosomes (Yarlett et al. 1984, 1985). Certain rumen ciliates utilize cellulose and starch – others predate on bacteria and smaller protozoa. Glucose is the major monosaccharide liberated by degradation of plant polymers and can be used by these rumen protozoa as fermentation substrate. The main end products of the metabolism of exogenously added glucose as well as of intracellular amylopectine of rumen ciliates with hydrogenosomes are hydrogen, acetate, lactate, butyrate, and CO<sub>2</sub> (Yarlett et al. 1985; Ellis et al. 1991a,b,c). The ratio in which these end products are formed is influenced by O<sub>2</sub> and CO<sub>2</sub> concentrations similar to those present in the rumen. The investigated rumen ciliates are able to use oxygen as terminal electron acceptor. The nature of this terminal oxidase is still unknown, but cytochromes appear not to be involved. *Dasytricha ruminantium* is the best-studied rumen ciliate, but even the knowledge of the metabolism of this rumen ciliate is still far from complete. The enzyme used for the degradation of pyruvate to acetyl-CoA in this protozoon is suggested to be PFO, which has been identified tentatively in the hydrogenosomal fraction (Yarlett et al. 1981, 1982, 1985). This acetyl-CoA is the substrate for the hydrogenosomal formation of acetate, but seems also to be exported from the hydrogenosomes for the formation of butyrate (Yarlett et al. 1985; Ellis et al. 1991b). These aspects make this hydrogenosome of rumen ciliates very different from that of *Nyctotherus* (Fig. 12) and also different from the hydrogenosomes of *Trichomonas* (Fig. 3) and the anaerobic chytrids (Fig. 9).

The only other published metabolic studies on hydrogenosomes of ciliates deal with the free-living Plagiopylid ciliate *Trimyema* (Goosen et al. 1988, 1990; Holler and Pfennig 1991; Shinzato and Kamagata 2010). *Trimyema* consumes oxygen under micro-aerobic conditions and is reported to produce formate as the major end product with minor amounts of acetate and lactate (Goosen et al. 1990; Holler and Pfennig 1991). Under those micro-aerobic conditions, hydrogen and ethanol are not produced. Under strictly anaerobic conditions, however, ethanol is the main end product, while acetate, lactate, formate, and hydrogen are then formed in minor amounts (Goosen et al. 1990; Holler and Pfennig 1991). This pattern of anaerobic fermentation products resembles the one found in anaerobic chytridiomycete fungi (Boxma et al. 2004; see above). These fungi perform a bacterial-type mixed acids fermentation, using PFL for the degradation of pyruvate, instead of PDH or PFO, which is used by *N. ovalis* and Trichomonads, respectively. Albeit that no additional biochemical data are available and that no cell fractionation studies have been performed, it is likely that the Plagiopylids evolved a type of hydrogenosome that is clearly different from those of *Nyctotherus* and *Dasytricha*.

#### **7.4 Can the Methanogenic Symbionts Tell Us More about the Origin and Function of Ciliate Hydrogenosomes?**

As mentioned before and described by Fenchel and Finlay (2010) and Ushida (2010), anaerobic ciliates are frequently associated with symbiotic methanogens. The nature of the methanogenic symbionts supports the conclusion that different

ciliates host different types of hydrogenosomes. While *Nyctotherus* and *Metopus*, and also *Plagiopyla* and *Trimyema*, host different endosymbiotic methanogens (Fenchel and Finlay 1995, 2010; van Hoek et al. 2000b; Hackstein et al. 2002), certain rumen ciliates seem to host episymbiotic methanogens. Whether this episymbiotic association is specific and whether there is any rumen ciliate (except *Dasytricha* and *Isotricha*) with endosymbiotic methanogens is still a matter of debate (Fenchel and Finlay 1995; Tokura et al. 1999; Regensbogenova et al. 2004; Ushida 2010).

Because the methanogens (regardless of being endo- or episymbiotic) rely on substrates provided by the host, the properties of the endosymbiont might provide some information about the metabolic characteristics of the host. The group of Vogels and Stumm succeeded in cultivating a number of putative methanogenic endosymbionts from the anaerobic ciliates *Metopus striatus*, *Metopus contortus*, and *Plagiopyla nasuta*, from the amoeboflagellate *Psalteriomonas* (*Lyromonas*) *vulgaris* and the giant amoeba *Pelomyxa palustris* (van Bruggen et al. 1984, 1986, 1988; Goosen et al. 1988; see Fenchel and Finlay 1995 for more references and discussion). The conclusion from these studies was that certain endosymbionts were similar if not identical to well-known free-living methanogens, e.g., *Methanobacterium formicicum*. Only the putative endosymbiont from *M. contortus* seemed to represent a new type of methanogen, i.e., *Methanoplanus endosymbiosus*. The latter host, *Metopus*, belongs to the same taxon as *N. ovalis*, which makes it likely that this ciliate (*Metopus*) possesses a similar mode of pyruvate metabolism. The metabolic properties of the methanogenic endosymbiont *M. formicicum*, however, suggested that this methanogen might be capable of using other substrates besides hydrogen and CO<sub>2</sub>, e.g., formate (Dong et al. 1994). Notably, Narayanan et al. (2009) provided evidence for the presence of an acetoclastic *Methanosaeta* species as endosymbiont of *Metopus es* suggesting that methanogenic endosymbionts might be able to use acetate excreted by the hydrogenosomes. This argues again for the metabolic diversity among ciliate hydrogenosomes and their methanogenic endosymbionts. This metabolic diversity could provide additional arguments for multiple origins of the hydrogenosomes, but unfortunately, metabolic data of both hosts and symbionts are scarce.

## 7.5 Evolutionary Aspects

There exists a rather broad agreement that the anaerobic ciliates evolved secondarily from aerobic ancestors since several ciliate taxa comprise both aerobic and anaerobic species. Phylogenetic studies suggest that hydrogenosomes have arisen independently at least three to four times in ciliates (Fig. 10; Clarke et al. 1993; Embley and Finlay 1994; Embley et al. 1995, 2003; Fenchel and Finlay 1995; Hirt et al. 1998; Hackstein et al. 2001, 2002). The existence in *N. ovalis* and *Blastocystis* of a “missing link,” an organelle with characteristics of mitochondria as well as hydrogenosomes, demonstrates that hydrogenosome-bearing ciliates can evolve



from mitochondriate ciliates (Martin 2005; Ricard 2008; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Albeit that the patchy distribution of hydrogenosomes alone is not sufficient to prove multiple independent origins of ciliate hydrogenosomes, the existence of a missing link like the hydrogenosome of *N. ovalis* provides a clear scenario for the evolution of hydrogenosomes from mitochondria. Apparently, hydrogenosomes in ciliates can evolve “easily” by evolutionary tinkering from mitochondria in the course of the adaptation of their hosts to anaerobic/micro-aerobic environments. This happened several times independently in the evolution of ciliates – at least three independent origins are supported by the existence of three different types of hydrogenosomes in the few ciliates that have been studied so far.

It remained unclear until now whether or not all anaerobic ciliates possess hydrogenosomes, in particular those anaerobes that do not possess endosymbiotic methanogens. Theoretically, anaerobic ciliates might possess anaerobic mitochondria (Tielens et al. 2002), hydrogenosomes, or they could even have lost ATP-generating organelles completely. In this case, they most likely host mitochondrial remnants, mitosomes, just like *Giardia* and *Entamoeba* spp., which are completely dependent on cytosolic reactions for the production of ATP (see, e.g., Hackstein et al. 2006 and Hjort et al. 2010 for discussion). However, the presence of these elusive organelles has not been studied systematically and in more detail in ciliates so far – with a few remarkable exceptions to be discussed below.

It has already been addressed that at least among the rumen ciliates, species with mitosomes might exist, because there is evidence that certain rumen ciliates, such as *Entodinium simplex*, *Entodinium caudatum*, *Diploplastron affine*, *Ophryoscolex caudatus*, *Eremoplastron bovis*, and *Ostracodinium obtusum bilobum*, did not exhibit detectable hydrogenase activity in the particulate cell fraction (Yarlett et al. 1984). Also, electron microscopy did not reveal the presence of mitochondrial-shaped organelles or typical hydrogenosomes in certain species of rumen and marsupial gut ciliates; a systematic search for mitosomes, however, has not been performed (Williams and Coleman 1992; Cameron and O’Donoghue 2002b). The observation that also PFO and malate dehydrogenase (decarboxylating) activities (Yarlett et al. 1984) are not enhanced in the particulate cell fraction, together with a low cytoplasmic hydrogenase activity, might argue for the absence of hydrogenosomes and potentially for the presence of mitosomes. However, until now, there are no additional data that could support this speculation.

The adaptation to an anaerobic lifestyle with the aid of hydrogenosomes required the acquisition of an (oxygen-sensitive) hydrogenase. The evolution of fumarate respiration in *N. ovalis* shows that an adaptation to life in anaerobic environments can occur in steps – by evolutionary tinkering. Once anaerobiosis could be tolerated by the invention of fumarate respiration, it became possible to acquire a hydrogenase. The [FeFe] hydrogenase of *N. ovalis* most likely has been obtained by lateral gene transfer from anaerobic (sulphate-reducing) bacteria (Boxma et al. 2007; de Graaf et al. 2009). The peculiar 24 and 51 kDa subunits of this complex hydrogenase are paralogous to the corresponding proteins of the mitochondrial Complex I (which is functional in *N. ovalis*), and have a different (most likely beta

proteobacterial) origin. The acquisition of this hydrogenase obviously allows a fine tuning of the NADH pool, which is crucial for the maintenance of homeostasis under anaerobic conditions. Thus, *N. ovalis* not only turns out to be a missing link but also demonstrates that the adaptation to anaerobic environments can involve several steps to allow the evolution of multiple levels for the control of homeostasis.

## 8 Conclusions

In the recent years, it has become clear that there are many mitochondria that do not function as described in most biochemical textbooks (Tielens et al. 2002). Moreover, there are mitochondrion-related organelles such as hydrogenosomes, mitosomes, mitochondrial remnants, and mitochondrion-like organelles (Fig. 1; Hackstein et al. 2006). All these organelles exhibit a large diversity of metabolic properties (Tielens et al. 2002; Tielens and van Hellemond 2007; van der Giezen, 2009; Hjort et al. 2010). In this review, the diversity of hydrogenosomes, bona fide hydrogenosomes, and hydrogenosome/mitochondrion-like organelles has been described. It has been shown that the metabolism of all these organelles is rather different corroborating their independent evolution from mitochondria and mitochondrion-like organelles. Their diversity is the consequence of their independent evolution in different anaerobic niches from organelles that were already adapted to different aerobic environments. The diversity of the hydrogenosomes described here is remarkable. Since there are many such organelles awaiting a more detailed analysis, a even larger diversity of hydrogenosomes might be expected.

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